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Anaerobic digestion of citrus waste using two-stage membrane bioreactor

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Abstract. Anaerobic digestion is a promising method to treat citrus waste. However, the presence of limonene in citrus waste inhibits anaerobic digestion process. Limonene is an antimicrobial compound and could inhibit methane forming bacteria that takes a longer time to recover than the injured acid forming bacteria. Hence, volatile fatty acids will be accumulated and methane production will be decreased. One way to solve this problem is by conducting anaerobic digestion process into two stages. The first step is aimed for hydrolysis, acidogenesis, and acetogenesis reactions and the second stage is aimed for methanogenesis reaction. The separation of the system would further allow each stage in their optimum conditions making the process more stable. In this research, anaerobic digestion was carried out in batch operations using 120 ml-glass bottle bioreactors in 2 stages. The first stage was performed in free-cells bioreactor, whereas the second stage was performed in both bioreactor of free cells and membrane bioreactor. In the first stage, the reactor was set into 'anaerobic' and 'semi-aerobic' conditions to examine the effect of oxygen on facultative anaerobic bacteria in acid production. In the second stage, the protection of membrane towards the cells against limonene was tested. For the first stage, the basal medium was prepared with 1.5 g VS of inoculum and 4.5 g VS of citrus waste. The digestion process was carried out at 55°C for four days. For the second stage, the membrane bioreactor was prepared with 3 g of cells that were encased and sealed in a 3×6 cm^2 polyvinylidene fluoride membrane. The medium contained 40 ml basal medium and 10 ml liquid from the first stage. The bioreactors were incubated at 55°C for 2 days under anaerobic condition. The results from the first stage showed that the maximum total sugar under 'anaerobic' and 'semi-aerobic' conditions was 294.3 g/l and 244.7 g/l, respectively. The corresponding values for total volatile fatty acids were 3.8 g/l and 2.9 g/l, respectively. Methane production of citrus waste taken from the first stage under 'anaerobic' condition in membrane and free-cells bioreactors was 11.2 Nml and 7.2 Nml, respectively. Whereas, methane production of citrus waste taken from the first stage under 'semi- aerobic' condition in membrane and free-cells bioreactors was 8.8 Nml and 5.7 Nml, respectively. It can be seen

from the results of the first stage that volatile fatty acids from 'anaerobic' condition was higher than that of 'semi-aerobic' condition. The absence of oxygen provides the optimal condition for growth and metabolism of facultative and obligatorily anaerobic bacteria in the first stage. Furthermore, polyvinylidene fluoride membrane was able to protect the cells from antimicrobial compounds.

1. Introduction

In 2015, juice and marmalade production created around 30-36 million tons of citrus [1][2]. Without proper treatment, this waste causes health and environmental problems. Fortunately, citrus waste consists mostly of organic matter. This makes it suitable for treatment by anaerobic digestion, which produces methane that can be used as an energy source.

Digestion is a complex process. When a feedstock is placed in an anaerobic digester, the facultative bacteria (hydrolytic bacteria) break down the complex molecules, using up the oxygen in the feed. They will also use the oxygen in the air trapped inside the digester. In the second and third steps, the acidogen and acetogen bacteria form low molecular weight volatile fatty acids (VFA), as well as carbon dioxide and hydrogen. In the last step, the methanogenic bacteria convert volatile fatty acids into water, carbon dioxide, and methane. However, the hydrolytic and acetogenic bacteria are generally not as sensitive as methanogens and grow much faster. Hence, there would be accumulation of VFA that results in low pH and could kill methanogens. To further complicate the problem, citrus contains a flavour compound, limonene, that has an antimicrobial effect. Limonene can cause inhibition of methane production because the injured methanogens take a longer time to recover than the injured acidogen and acetogen bacteria. As a result, methane production will be decreased and VFA will be accumulated.

One way of solving the methanogen acidification problem is by applying two-stage anaerobic digestion [3]. The purpose of two-stage digestion is to separate the conditions for each stage, *i.e.* acid formation (1st stage) and methane formation (2nd stage). Two-stage anaerobic digestion provides optimal conditions for each stage, such as pH, temperature, and alkalinity, to achieve optimal growth of digesting bacteria. It is also suggested [4] that digestion of citrus waste be conducted using a membrane bioreactor in which cells are encased in a hydrophilic membrane. Hence, limonene, being a hydrophobic compound, will be prevented from accessing the cells. In this work, a membrane bioreactor was applied in the 2nd stage of digestion to protect the cells from limonene in the citrus waste.

2. Methodology

2.1. Inoculum

The inoculum was obtained from a thermophilic biogas plant (55°C) (Borås Energy and Environment AB, Borås, Sweden). It was kept at 55°C for three days to acclimatize the cells and to settle the sludge. The acclimated sludge was homogenized, filtered through a sieve with a pore size of 1.0 mm in order to remove the remaining large particles. It was then centrifuged (Heraus Megafuge 8, Thermo Scientific, Germany) at 1649g for 20 minutes to separate the supernatant and the suspended sludge. The suspended sludge was later used as an inoculum either as free cells or cell containment in membrane sachets.

2.2. Preparation of membrane cell

Membrane cell was prepared by inserting 3 g of the suspended sludge of inoculum into a sachet made from a hydrophilic polyvinylidene fluoride (PVDF) membrane (Durapore®, Thermo Fisher Scientific Inc., Sweden) [4]. The membrane sachet was prepared by cutting the membrane into a size of 3 cm x 6 cm and sealed on three sides of the membrane with heating. After the insertion of the cells, the sachet was sealed to close. The membrane cells were immediately used for the experiment.

2.3. Citrus wastes

The citrus wastes were supplied by Brämhult juice AB (Borås, Sweden) and stored at - 20°C before use. The wastes were from orange juice process and contained in (%db): total solid (TS) (23.9), volatile solid (VS) (23.1), carbohydrate (18.5), protein (1.28), and fat (0.23). The citrus wastes were thawed and grounded to achieve a size of around 20 mesh before use.

2.4. Medium

The basal medium was added to supply necessary nutrients for the microorganism. The composition of the basal medium was as follows (in mg/l): NH_4Cl (280), $K_2HPO_4.3H_2O$ (330), $MgSO_4.7H_2O$ (100) and $CaCI_2.2H_2O$ (10). One millilitre of trace nutrient solution was added per litre of basal medium. The trace nutrient solution contained (in mg/l): $FeCl_2.4H_2O$ (2000), H_3BO_3 (50), $ZnCl_2$ (50), $MnCl_2.4H_2O$ (500), $CuCl_2.2H_2O$ (38), $(NH_4)_6$ $Mo_7O_{24}.4H_2O$ (50), $CoCl_2.6H_2O$ (2000), $NiCl_2.6H_2O$ (142), $Na_2SeO_3.5H_2O$ (164).

2.5. Anaerobic Digestion

2.5.1 First Stage. A 500-ml glass bottle bioreactor was used as a batch digester with a total working volume of 250 ml. Each bioreactor contained 4.5 g VS of ground citrus waste and 1.5 g VS of free cells of inoculum. The basal medium was added until the working volume was achieved. There was no pH adjustment for the basal medium in the 1st stage. The reactors were then closed and incubated under either 'anaerobic' or 'semi-aerobic' conditions at 55°C for 4 days. To obtain 'anaerobic condition', the bioreactor was flushed with a gas mixture of 80% nitrogen (N_2) and 20% carbon dioxide (CO_2). To obtain 'semi-aerobic condition', the bioreactor was injected with 100 ml air/day. The bioreactors were closed with rubber septum and green cap (GL 45). The digesters were shaken manually each day. Control sample containing only citrus waste and basal medium was prepared in order to investigate whether citrus waste produced VFA and methane without the inoculum. Moreover, inoculum and basal medium were applied as a blank in order to measure gas production from the inoculum alone.

2.5.2 Second Stage. A 120-ml glass bottle bioreactor was used as a batch digester with a total working volume of 50 ml. The glass digester was filled with 10 ml of liquid from the first stage of digestion, 40 ml of basal medium, and 3 g of free cells or encased cells. For the 2nd stage, the pH of the basal medium was adjusted to 7. A glass digester containing only 3 g of inoculum and 50 ml basal medium was prepared as a blank. The glass digester was tightly sealed with 20 mm aluminium crimp cap with inserted grey PTFE/butyl rubber septum. The glass digesters were incubated under 'anaerobic condition' at 55°C for 2 days in the incubator and shaken manually each a day. The experimental set up are shown in Figure 1.

All the experiments of the 1st stage and the 2nd stage were run in triplicate and the results were shown as an average.

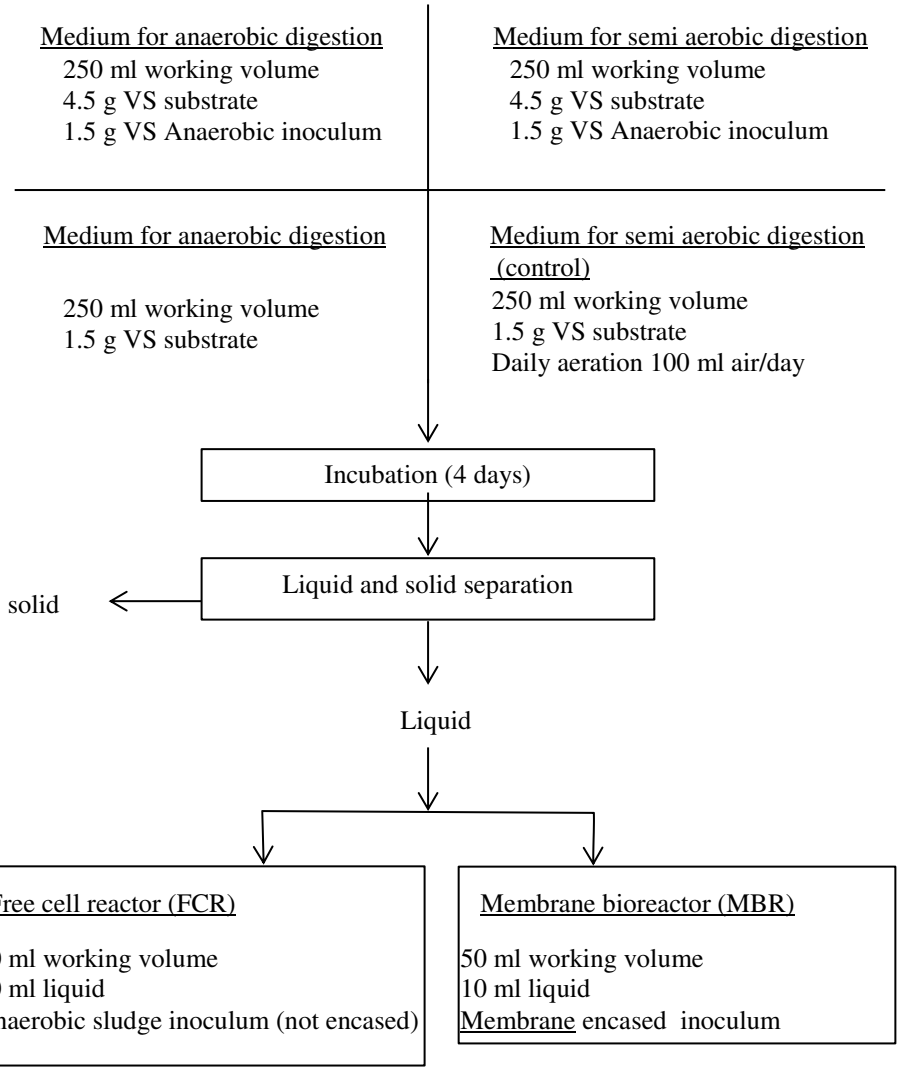

Figure 1. Experimental set up of first and second stage digestion

2.6. Analysis

Volatile fatty acids (VFA) including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and total sugar including glucose, sugar mix were analyzed using a High Performance Liquid Chromatography (HPLC) (Waters, USA) that was equipped with an ion-exchange column (Aminex HPX-87H, Biorad, Richmond, CA, USA). It was operated at 50°C, with a UV-VIS detector (Waters 486, Milipore, Milford, MA) at 210 nm with 5 mM sulfuric acid solution (Sigma Aldrich) as an eluent at a flow rate of 0.6 ml/min. Prior to analysis, samples were first filtered using syringe filter (Acrodisc 32 mm with 0.2 μm support membrane).

VS dissolution of the substrate was determined by subtracting VS of the substrate in the end experiment with VS of the substrate in the 1st-day experiment. Volatile solid content in the beginning and in the end experiment were determined [5].

Methane production was measured using a Gas Chromatograph (Varian 450 GC, U.S.A.) with a capillary column equipped with a thermal conductivity detector (TCD). N_2 was used as a carrier gas, and the instrument was operated with injector and oven temperature at 75°C and 50°C, nitrogen column flow 2.0 ml/min and detector temperature at 250°C. A 0.25 ml pressure lock syringe (VICI, U.S.A.) was used for the gas sampling.

3. Results and discussion

In this work, two-stage anaerobic digestion of citrus waste was applied in order to overcome acidification problem that is potential to inhibit methane production. Several parameters including sugar production, volatile fatty acid (VFA) production, methane production, pH value, and volatile solid (VS) dissolution were observed.

3.1. Production of total sugar and volatile fatty acids in the 1st stage of anaerobic digestion of citrus waste

Sugar is one of the hydrolysis step products. In the hydrolysis step, the complex organic matters, such as carbohydrate, protein, and fats are hydrolyzed into their monomers such as sugars, amino acids, and fatty acids. In this study, only total sugar production that was observed because the main chemical composition of citrus waste was carbohydrate. The total sugar production of anaerobic digestion of citrus waste is presented in Figure 2. Total sugar production from the digestion of citrus waste in all conditions continued to increase but started to decrease after the 1st day and reached a plateau since the 2nd day of digestion. The reason for this could be due to the facts that all the substrate was already converted to sugars and the sugars were converted to volatile fatty acids. It can be observed that the highest concentration of total sugar from the digestion under 'anaerobic condition' was bigger than that of under 'semi-aerobic condition'. The concentration of total sugar under 'anaerobic' and 'semi-aerobic' conditions was 294.3 g/l and 244.7 g/l, respectively. Microorganisms of many different genera are responsible for hydrolysis, most of them are facultative anaerobic bacteria and obligatory anaerobic bacteria [6]. The results of this work showed the presence of oxygen in 'semi- aerobic' condition decreased production of sugar.

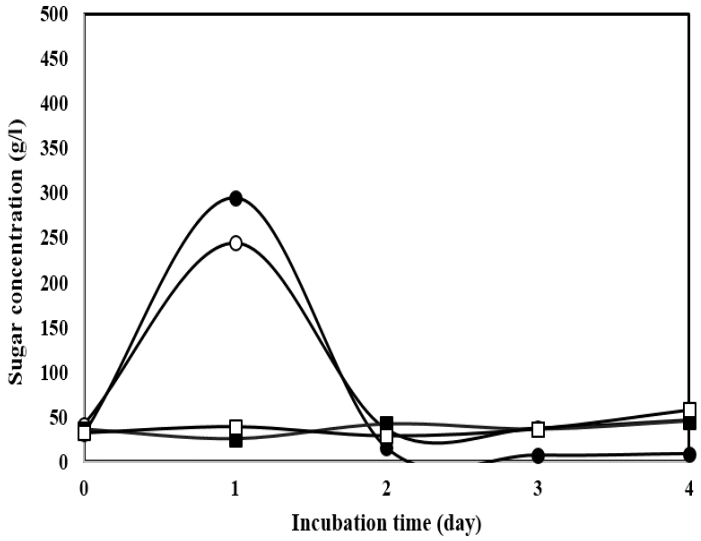

Figure 2. Sugar production of citrus waste anaerobic condition (●), semi-aerobic condition (❍), control anaerobic condition (■) and control semi-aerobic condition (□) for 4 days.

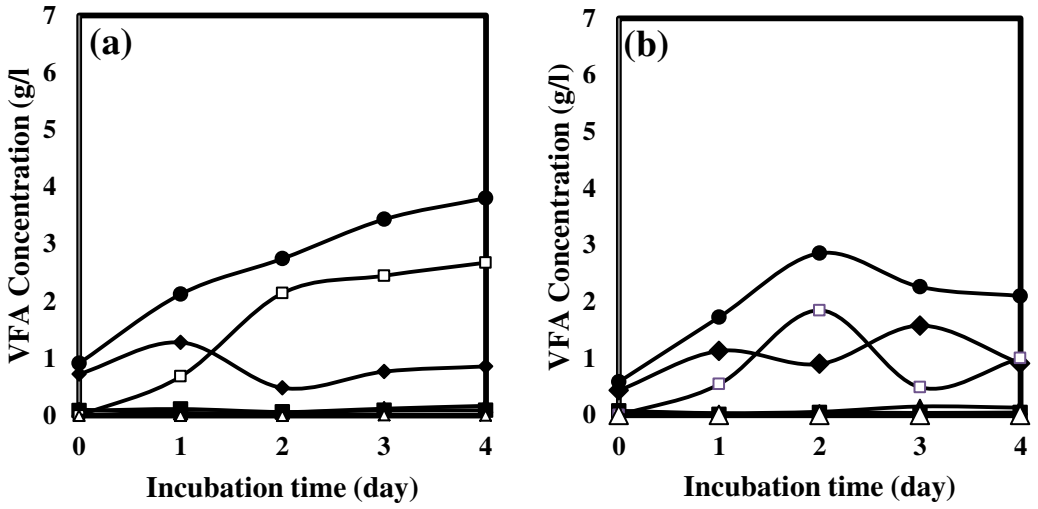

Figure 3. Volatile fatty acids (VFA) profile of citrus waste added with inoculum in anaerobic condition (a) and semi-aerobic condition (b). Concentration of total VFA (●), acetic acid (♦), propionic acid (■), isobutyric acid (▲), butyric acid (□) and isovaleric acid (△) for 4 days of incubation.

Figures 3(a) and 3(b) show the profile of concentration of total VFA as well as the individual VFA including acetic acid, propionic acid, isobutyric acid, butyric acid, and isovaleric acid. Volatile fatty acids are precursors of methane, therefore the production of VFA is necessary to be observed. Under 'anaerobic' condition, the production of VFA continued to increase until the last day of digestion. Whereas under 'semi-aerobic condition', VFA production already reached nearly a constant level after day-2. Concentration of VFA that has a longer chain than butyric acid was constantly low. In the 'semi-aerobic' condition, some of the butyric acid was converted to acetic acid at early stage. This is probably due to some of the acetogenic bacteria that are facultatively anaerobic. The concentration of butyric acid decreased and the concentration of acetic acid increased after the 1st day. As a result, the concentration of total VFA was almost constant. If Figure 3(a) and Figure 3(b) were compared, production of VFA from the digestion of citrus waste under 'anaerobic condition' was higher than that of 'semi-aerobic condition'. The production of VFA results in the decrease of pH of the medium. Initially, the pH of the medium was 6.6-7.3. After digestion for 4 days, the pH dropped to 4.7-4.9.

The volatile solid (VS) dissolution by microorganisms was also measured to investigate the amount of organic materials consumed to produce sugar and VFA by the digesting bacteria. The VS dissolution of citrus waste in 'anaerobic' condition and 'semi-aerobic' condition was 63.46%, 59.29%, respectively. This result is relevant to the VFA production. The highest VFA production during four days of digestion of citrus waste in 'anaerobic' and 'semi-aerobic' was 3.8 g/l, 2.9 g/l, respectively. The greater the dissolution of volatile solid, the higher the production of VFA. After anaerobic digestion was run for 4 days, the process was stopped. The liquid and the solid part was separated and the liquid part was used as substrate for the 2nd stage to produce methane.

3.2. Production of methane in the 2nd stage of anaerobic digestion of citrus waste

The 2nd phase of anaerobic decomposition is dominated by a group of methanogenic bacteria, which grow in neutral condition (pH 6.7-7.5) [7]. Thus, the pH of the 2nd stage medium was adjusted to 7 as the substrate (the liquid part) from the 1st stage had a low pH. One of the indicators of digestion

process is pH value. A drop in pH value is an indication of a disturbance of the fermentation process [6]. The measurement of the pH value in the medium in all experiments on the last day of digestion showed the value of around 7. This means that methanogens were growing in their appropriate condition.

Figure 3(a) and Figure 3(b) show that methane was produced from the liquid taken from the 1st stage both under 'anaerobic' and 'semi-aerobic' conditions, respectively. To evaluate the performance of MBR, the methane production was compared with bioreactor using only free cells as inoculum (FCR). Anaerobic digestion of liquid from the 1st stage under 'anaerobic' condition produced higher methane compared to that of 'semi-aerobic' condition. As the VFA in the liquid from semi-aerobic condition was lower than anaerobic condition, accordingly, it can be understood that the methane production of the liquid from semi-aerobic condition was also lower.

Furthermore, the methane was produced higher by using MBR system than FCR system. The accumulated methane production in MBR and FCR systems under 'anaerobic' condition were 11.2 Nml and 7.2 Nml, respectively. The corresponding values under 'semi-aerobic' were 8.8 Nml and 5.7 Nml, respectively. The result indicates that the methane-producing bacteria encased in PVDF membrane were better protected against any harmful compound present in the medium. It is well known that citrus waste contains 0.5% peel oil and more than 90% of it is an antibacterial component, named-limonene. Limonene at concentration of 400 μL/L was reported to inhibit the anaerobic digestion process and caused ultimate failure during continuous operation at mesophilic conditions [8]. According to the biochemical reaction series of anaerobic digestion, methane is formed from acetate (by acetogenic methanogens) and H2/CO2 (by hydrogenotrophic methanogens) [6]. Limonene was found to be inhibitory more for acetogenic methanogens rather than hydrogenotrophic methanogens [9]. Acetic acid consumption was inhibited in the presence of a limonene concentration of 9.10-4 M. Limonene being hydrophobic was prevented diffusing into the hydrophilic membrane. As a result, the cells capacity against inhibition effect is enhanced. Previous study reported that no methane was produced on the first 10 days of anaerobic digestion of orange peel using single stage digestion with FCR system [10]. The methane started to be produced on day-20. The result indicated that digestion of citrus waste on single stage FCR system faced a long lag phase. On the contrary, application of two-stage membrane bioreactor in this work resulted in 8.8-11.2 mL of methane on day-2 (in the 2nd stage) and no lag phase was observed.

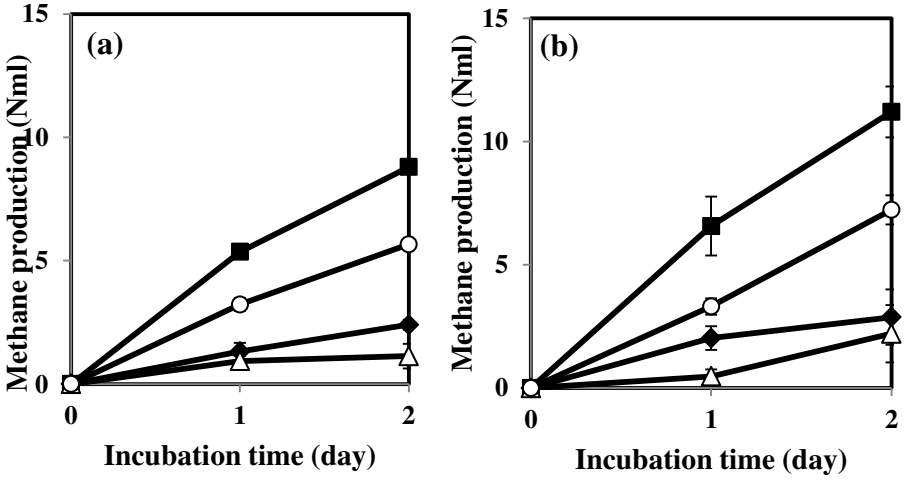

Figure 4. Methane production in citrus waste 'semi-aerobic' condition (a), 'anaerobic' condition (b) for blank MBR (♦), control FCR (△), citrus waste FCR (❍) and citrus waste MBR (■) for two days.

4. Conclusion

- Volatile fatty acids (VFA) produced from the digestion of citrus waste under 'anaerobic condition' was higher than that of under 'semi-aerobic', The higher metabolite products production from the digestion under anaerobic condition indicates that the presence of oxygen in 'semi-aerobic' inhibited metabolism of the microorganisms in the 1st stage.
- Two-stage anaerobic digestion could minimize lag phase of methane production from the digestion of citrus waste

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